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Dated: October 3, 2007 Signature: _____ (Jeffrey S. Sharp)

Docket No.: 13024/38627A
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
John McMichael et al.

Application No.: 10/624,328

Confirmation No.: 6871

Filed: July 22, 2003

Art Unit: 1614

For: METHOD OF TREATMENT OF PSYCHOLOGICAL CONDITIONS BY ADMINISTRATION OF NERVE GROWTH FACTOR

Examiner: D. C. Jones

APPEAL BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This Appeal Brief is submitted in support of the Notice of Appeal, mailed in this application on June 4, 2007. This Appeal Brief is accompanied by the fee for filing an Appeal Brief under 37 C.F.R. §1.17(b) and a two-month extension of time under 37 C.F.R. §1.136(a). Accordingly, this Appeal Brief is timely filed and no further fees are believed due. Any additional required fee may be charged, or any overpayment credited, to Deposit Account No. 13-2855.

TABLE OF CONTENTS

This brief contains items under the following headings as required by 37 C.F.R. § 41.37 and M.P.E.P. § 1205.2:

I. REAL PARTY IN INTEREST

The real party in interest for this appeal is Milkhaus Laboratory, Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals, interferences, or judicial proceedings which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS

A. Total Number of Claims in Application

There are 20 claims pending in application.

B. Current Status of Claims

1. Claims canceled: 3, 6-7, 19
2. Claims withdrawn from consideration but not canceled: None
3. Claims pending: 1, 2, 4, 5, 8-18 and 20-24
4. Claims allowed: None
5. Claims rejected: 1, 2, 4, 5, 8-18 and 20-24

C. Claims On Appeal

The claims on appeal are claims 1, 2, 4, 5, 8-18 and 20-24.

IV. STATUS OF AMENDMENTS

No amendments to the application were made after the Final Rejection dated January 22, 2007. All previous amendments have been entered and are reflected in the pending claims, set forth in Appendix A.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention is directed towards methods of treating psychological conditions.

Independent claim 1 is directed to a method of alleviating symptoms of a psychological condition such as depression, anxiety disorders, panic attacks, premenstrual dysphoric disorder (PMDD), and premenstrual syndrome (PMS) and comprises the step of administering to a subject in need thereof an effective amount of nerve growth factor (Specification page 13, line 29 through page 14, line 8).

Independent claim 15 is directed to a method of alleviating symptoms of a psychological condition selected from the group consisting of sleep disorders, tension headaches, and constipation comprising administering to a patient in need thereof nerve growth factor in an amount effective to treat one or more of said symptoms (Specification page 14, lines 19-16).

Claims 2, 4, 5, 8 and 9 each depend from claim 1 and further recite that said psychological condition is an depression, an anxiety disorder, a panic attack, premenstrual dysphoric disorder (PMDD) or premenstrual syndrome (PMS) (Specification page 14, lines 5-8).

Claims 10 and 20 depend from claims 1 and 15, respectively, and additionally recite, that said nerve growth factor is administered by a mode selected from the group consisting of sublingual, bucal, oral drench, subcutaneous, intradermal or intravenous (Specification page 14, lines 16-18 and 31 and page 15, lines 1-2).

Claims 11 and 21 depend from claims 10 and 20, respectively, and recite that the nerve growth factor is administered sublingually (Specification page 14, lines 17 and 31).

Claims 12 and 22 depend from claims 1 and 15, respectively, and additionally recite that the nerve growth factor is administered in an amount ranging from about 0.001 to 1 μg per day (Specification page 14, lines 9 and 24).

Claims 13 and 24 depend from claims 1 and 15, respectively, and additionally recite that the nerve growth factor is administered in an amount ranging from of 0.01 to 0.1 μg per day (Specification page 14, lines 16 and 30-31).

Claim 14 depends from claim 1 and additionally recites that the symptoms are selected from a group consisting of sleep disorders, tension headaches, cold sweats, and constipation (Specification page 14, lines 20-21).

Claims 16-18 each depend from claim 15 and further recite that the symptom is a sleep disorder, a tension headache and constipation, respectively (Specification page 14, lines 20-21).

Claim 23 depends from claim 15 and additionally recites that the nerve growth factor is administered in an amount raging from 0.05 to 1 μg per day (Specification page 14, line 29).

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

A. Whether claims 1, 2, 4, 5, 8-18 and 20-24 are unpatentable under 35 U.S.C. § 103(a) over Frey II (U.S. Patent Application Publication No. 2003/0072793) in view of Beers (The Merck Manual of Diagnosis and Therapy, 17th Edition, pp 1525-1539 and pp 1932-1933, 1999).

B. Whether claims 1, 2, 4, 5, 8-18 and 20-24 are unpatentable under 35 U.S.C. § 103(a) over Siuciak (U.S. Patent No. 5,599,560) in view of Beers (The Merck Manual of Diagnosis and Therapy, 17th Edition, pp 1525-1539 and pp 1932-1933, 1999).

VII. ARGUMENT

Appellants submit that the rejections issued in the final Office Action are in error, and that the present application is in condition for allowance. Appellants respectfully request the Board to review and reverse each of the rejections issued in the final Office Action.

A. Frey II is directed to a mode of administering therapeutic agents to the central nervous system (CNS) and the rejection of claims 1, 2, 4, 5, 8-18 and 20-24 as assertedly being unpatentable under 35 U.S.C. § 103(a) should be reversed because the combined disclosure of Frey II and Beers does not specifically teach a method of treating any disorder by administration of nerve growth factor (NGF).

The rejection of claims 1, 2, 4, 5, 8-18 and 20-24 should be reversed because neither of Frey II, nor Beers, nor the combined disclosure of Frey II and Beers specifically discloses or suggests the administration of NGF for the treatment of any disorder.

Frey II teaches that a variety of therapeutic agents can be administered to the central nervous system (CNS) of a subject by way of a tissue innervated by the trigeminal nerve that is outside of the nasal cavity and is not directed to any specific treatment for any particular disease. Frey II discloses a laundry list of more than forty (40) potential agents for delivery to the CNS of a subject by a particular mode of drug administration, but fails to disclose or suggest that any one of the forty-plus agents disclosed therein is for the treatment of any one disorder. The list of the forty-plus agents (see Frey II, paragraphs [0036]-[0104]) that could potentially be administered by the method disclosed in Frey II and the list of conditions (See, Frey II, paragraph [0169]) for which it might be desirable to directly administer drugs to the CNS is not prescriptive.[1] Indeed, most of the forty-plus agents listed would not be expected to be therapeutic for most of the seventeen or more conditions listed in paragraph [0169]. For example, one would not expect that an "anti-cancer agent" (Frey II, paragraph [0045]) would be therapeutic for an anxiety disorder (Frey II, paragraph [0169]). Similarly, one would not expect that cisplatin, an anti-viral agent, (Frey II, paragraph [0043]) would be therapeutic for attention deficit disorder (Frey II, paragraph [0169]). Moreover, some combinations of agent and condition disclosed in Frey II may be contraindicated.

[1] There are at least 680 different therapeutic agent/CNS disorder combinations implied by the Examiner's argument (See Office Actions mailed April 27, 2006 and January 22, 2007).

In view of Frey II's failure to specifically correlate any particular agent as a therapeutic for a specific disorder, one of skill in the art would not know which of the many agents disclosed in Frey II could be used for the treatment of, for example, affective disorders. Therefore, one of skill in the art would not be motivated upon review of Frey II to use NGF for the treatment of any disorder, much less the psychological disorders or symptoms recited in independent claims 1 and 15, respectively.

Beers is a text book for the diagnosis and therapy of various disorders but fails to make up for the deficiencies of Frey II because it does not disclose or suggest the use of NGF for the treatment of any disease. In fact, Beers makes no mention of NGF at all! Therefore, the combined disclosure of Frey II and Beers fails to disclose or suggest the claimed invention. Accordingly, there can be no *prima facie* case of obviousness.

In hindsight, it is now known that NGF can be used to alleviate the symptoms a psychological condition, but there was no teaching in the art relied upon by the Examiner that one would be successful in doing so prior to Appellants' disclosure. The only motivation or suggestion to use NGF to alleviate the symptoms of a psychological disorder arises out of the instant application and it is impermissible to use the Appellants' own disclosure to find a motivation for the claimed invention, as this would be the epitome of hindsight reconstruction. See, M.P.E.P. § 2141.

For these reasons, Appellants submit that the Examiner has failed to establish a *prima facie* case of obviousness for the subject matter of any of claims 1, 2, 4, 5, 8-18 and 20-24 under 35 U.S.C. § 103(a) over Frey II in view of Beers. Accordingly, the rejection should properly be reversed.

B. Siuciak teaches the use of BDNF, NT-3 and NT-4 to treat depression but the rejection of claims 1, 2, 4, 5, 8-18 and 20-24 as being unpatentable under 35 U.S.C. § 103(a) over Siuciak in view of Beers should be reversed because nerve growth factor (NGF) is a different protein than BDNF, NT-3 and NT-4 and NGF is known to have different properties than BDNF, NT-3 and NT-4.

The rejection of claims 1, 2, 4, 5, 8-18 and 20-24 should be reversed because NGF is not brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) or neurotrophin-4 (NT-4).

NGF is a different protein with different properties than BDNF, NT-3 and NT-4 disclosed by Siuciak for treatment of depression. In fact, Siuciak itself emphasizes that NGF lacks some of the biological properties of BDNF, NT-3 and NT-4 (col. 3, lines 11-14) contradicting the Examiner's apparent assertion that all members of the neurotrophin family can be used interchangeably.

At column 3, lines 11-14, Siuciak refers to a study by Friedmann et al. (Exp. Neph., 119:72-78, 1999, submitted in response to the Final Office action mailed January 22, 2007 and submitted herewith in Appendix B as Exhibit C) which reports that members of the NGF family of neurotrophins exhibit different biological activities. (See, for example, Friedmann et al. page 76, second column, first paragraph).

> Specific neurotrophins such as NT-3 and NT-4 enhanced survival of both neuronal populations [locus coeruleus (LC) and basal forebrain (BF)]. **NGF, which has no effect on LC neurons,** influenced the BF by increasing cholinergic function but not survival, **in contrast to BDNF, NT-3, and NT-4.** (page 76, second column, first full paragraph, emphasis added.)

Accordingly, one of skill in the art would not have concluded from Siuciak or any other cited reference that NGF would be expected to have the same biological activities as BDNF, NT-3 and NT-4. Similarly, one of skill in the art would not have concluded from Siuciak that NGF could be used as a therapeutic in disorders normally treated with BDNF, NT-3 or NT-4 and would not be motivated upon review of Siuciak to use NGF to alleviate the symptoms of the psychological disorders or any other disease.

Beers is a text book for the diagnosis and therapy of various disorders but fails to make up for the deficiencies of Siuciak because it does not disclose or suggest the use of NGF for the treatment of <u>any</u> disease. In fact, Beers makes no mention of NGF at all! Therefore, the combined disclosure of Siuciak and Beers fails to disclose or suggest the claimed invention. Accordingly, there can be no *prima facie* case of obviousness.

In hindsight, it is now known that NGF can be used to alleviate the symptoms a psychological condition, but there was no teaching in the art relied upon by the Examiner that one would be successful in doing so prior to Appellants' disclosure. The only motivation or suggestion to use NGF to alleviate the symptoms of a psychological disorder arises out of

the instant application and it is impermissible to use the Appellants' own disclosure to find a motivation for the claimed invention, as this would be the epitome of hindsight reconstruction. See, M.P.E.P. § 2141.

For the foregoing reasons, Appellants submit that the Examiner has failed to establish a *prima facie* case of obviousness for the subject matter of any of claims 1, 2, 4, 5, 8-18 and 20-24 under 35 U.S.C. § 103(a) over Siuciak in view of Beers. Accordingly, the rejection may properly be reversed.

VIII. CLAIMS

A copy of the claims involved in the present appeal is attached hereto as Appendix A. As indicated above, the claims in Appendix A include the amendments filed by Appellants on January 3, 2006.

IX. EVIDENCE

There is no evidence pursuant to §§ 1.130, 1.131, or 1.132. Evidence entered by the Examiner is attached to Appendix B as Exhibits A and B and C.

X. RELATED PROCEEDINGS

There are no related proceedings.

Dated: October 3, 2007

Respectfully submitted,

By _____

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APPENDIX A

Claims Involved in the Appeal of Application Serial No. 10/624,328

1. (Previously presented) A method of alleviating symptoms of a psychological condition selected from the group consisting of depression, anxiety disorders, panic attacks, premenstrual dysphoric disorder (PMDD), and premenstrual syndrome (PMS) comprising administering to a subject in need thereof nerve growth factor in an amount effective to treat one or more symptoms of said psychological condition.

2. (Original) The method of claim 1, wherein said psychological condition is depression.

3. (Canceled)

4. (Original) The method of claim 1, wherein said psychological condition is an anxiety disorder.

5. (Original) The method of claim 1, wherein said psychological condition is panic attacks.

6-7. (Canceled)

8. (Original) The method of claim 1, wherein said psychological condition is premenstrual dysphoric disorder (PMDD).

9. (Original) The method of claim 1, wherein said psychological condition is premenstrual syndrome (PMS).

10. (Original) The method of claim 1, wherein said nerve growth factor is administered by a mode selected from the group consisting of sublingual, bucal, oral drench, subcutaneous, intradermal, or intravenous.

11. (Original) The method of claim 10, wherein said nerve growth factor is administered sublingually.

12. (Original) The method of claim 1, wherein said nerve growth factor is administered at a daily dosage of/from 0.001 to 1 microgram per day.

13. (Original) The method of claim 1, wherein said nerve growth factor is administered at a daily dosage of from 0.01 to 0.1 microgram per day.

14. (Previously presented) The method of claim 1, wherein the symptoms are selected from a group consisting of sleep disorders, tension headaches, cold sweats, and constipation.

15. (Previously presented) A method of alleviating symptoms of a psychological condition selected from the group consisting of sleep disorders, tension headaches, and constipation comprising administering to a patient in need thereof nerve growth factor in an amount effective to treat one or more said symptoms.

16. (Original) The method of claim 15, wherein said symptom is a sleep disorder.

17. (Original) The method of claim 15, wherein said symptom is a tension headache.

18. (Original) The method of claim 15, wherein said symptom is constipation.

19. (Canceled)

20. (Original) The method of claim 15, wherein said nerve growth factor is administered by a mode selected from the group consisting of sublingual, bucal, oral drench, subcutaneous, intradermal, or intravenous.

21. (Original) The method of claim 20, wherein said nerve growth factor is administered sublingually.

22. (Original) The method of claim 15, wherein said nerve growth factor is administered at a daily dosage of/from 0.001 to 10 micrograms per day.

23. (Original) The method of claim 15, wherein said nerve growth factor is administered at a daily dosage of/from 0.05 to 1 micrograms per day.

24. (Original) The method of claim 15, wherein said nerve growth factor is administered at a daily dosage of/from 0.01 to 0.1 micrograms per day.

25-28. (Canceled)

APPENDIX B

EVIDENCE RELIED UPON PURSUANT TO §§ 1.130, 1.131, OR 1.132

There is no evidence pursuant to §§ 1.130, 1.131, or 1.132.

EVIDENCE RELIED UPON BY THE EXAMINER:

Copies of the Frey II U.S. Patent Publication No. 2003/0072793, Siuciak U.S. Patent No. 5,599,5602 and Beers, M.H.and Berkow, R., The Merck Manual of Diagnosis and Therapy, 17th Edition, pages 1525-1539 and 1932-1933, 1999, references relied upon by the Examiner are attached hereto as Exhibits A and B. Attached as Exhibit C is a copy of Friedmann et al., Exp. Neph., 119:72-78, 1999, which Appellants submitted in response to the Final Office action mailed January 22, 2007.

APPENDIX C

No related proceedings are referenced in Section II above, hence copies of decisions in related proceedings are not provided.

EXPERIMENTAL NEUROLOGY 119, 72–78 (1993)

Differential Actions of Neurotrophins in the Locus Coeruleus and Basal Forebrain

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The neurotrophin gene family, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/NT-5, supports the survival of distinct peripheral neurons, however, actions upon central neurons are relatively undefined. In this study we have compared different neurotrophins in the regulation of neuronal survival and function using dissociated embryonic cell cultures from two brain regions, the basal forebrain (BF) and locus coeruleus (LC). In the BF, NGF increased choline acetyl transferase (ChAT) activity, but did not influence cholinergic cell survival. In contrast to NGF, BDNF, NT-3, and the novel neurotrophin, NT-4, all increased ChAT activity and cholinergic cell survival. We also examined embryonic LC neurons in culture. LC neurons are unresponsive to NGF. In contrast, NT-3 and NT-4 elicited significant increases in survival of noradrenergic LC neurons, the first demonstration of trophic effects in this critical brain region. Identification of factors supporting coeruleal and basal forebrain neuronal survival may provide insight into mechanisms mediating degeneration of these disparate structures in clinical disorders. © 1993 Academic Press, Inc.

INTRODUCTION

Factors which regulate neuronal survival may be of critical importance for normal development and mature function of the central nervous system (2). A variety of data suggests that target-derived factors play a critical role in the survival of afferent neurons. The neurotrophin family of neurotrophic factors, which includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4, are all expressed in the hippocampus in the adult and at various stages of development. In the present studies we characterize the influence of the different neurotrophins on two afferent populations, the basal forebrain cholinergic neurons and the locus coeruleus noradrenergic population.

The most extensively characterized neurotrophic factor, NGF, has been shown to influence cholinergic function of basal forebrain (BF) neurons by increasing activity of choline acetyltransferase (ChAT), the acetylcholine-synthesizing enzyme (13, 31, 41). Further, infusion of NGF after fimbria–fornix transection prevents cholinergic cell death (16, 15, 33, 43), indicating that NGF may influence neuronal survival after a lesion. Damage itself may induce responsivity to NGF in adult basal forebrain neurons; cholinergic neurons are influenced by NGF after fimbria transection, but not in unlesioned adult rats (42, 16) or after removal of the hippocampal target without transection of the pathway (39). Thus, the role of NGF in supporting cholinergic cell survival in the normal, unlesioned brain is unclear. Responsivity of BF cholinergic neurons to NGF may depend on the physiological state of the neurons, either during a particular developmental period or after trauma.

NGF is now known to be a member of a neurotrophin gene family consisting of BDNF (28), NT-3 (19, 30, 7, 38), NT-4 (14), and NT-5 (3). The last appears to represent the mammalian homologue of NT-4 (23), first isolated from *Xenopus laevis* (14). BDNF and NT-3 are expressed in the hippocampus in overlapping but distinct patterns (9, 35). Moreover, BDNF influences BF cholinergic neurons and may support cholinergic innervation to the hippocampus (1, 26). NT-3-responsive neuronal populations remain undefined, although trophic effects have been observed in the BF (1). Expression of this factor in the brain is restricted to the hippocampus and certain cortical regions (11, 9, 35). The novel neurotrophin, NT-4/NT-5, is expressed at low levels in the adult rat brain (3), particularly in cerebral cortex and hippocampus (unpublished results), although potential functions in the mammalian brain are unknown.

We have examined the influence of specific neurotrophins on neuronal survival in the basal forebrain and locus coeruleus. Characterization of trophic requirements may provide insight into mechanisms mediating neuronal survival in these two brain regions which project to a common target.

METHODS

Brain Cultures

Time-pregnant Sprague–Dawley rats (Taconic Laboratories) were housed in clear plastic cages with *ad lib.*

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access to Purina Lab Chow and water. Animals were exposed to fluorescent illumination between 5 AM and 7 PM daily. Embryonic age was calculated from the day of discovery of the vaginal plug, which was Embryonic Day 1 (E1).

At E16 pregnant rats were sacrificed by exposure to CO_2 and soaked in 80% ethanol for 10 min. Fetuses were removed under sterile conditions and kept in PBS on ice for microscopic dissection of basal forebrain or the rostral rhombencephalon including the locus coeruleus (6). The tissue was dissociated by trituration and plated on polylysine-coated tissue culture dishes at a density of 1 million cells per 35-mm dish (Corning). The cells were maintained for 4 or 7 days in either serum-containing medium (Eagle's MEM, 7.5% FCS, 6 g/l glucose, penicillin–streptomycin), which promotes survival and proliferation of non-neuronal support cells, or in a serum-free medium which yields a relatively neuron-pure culture. The serum-free medium was composed of MEM and Ham's F-12 (1:1), glucose (6 mg/ml), insulin (25 μg/ml), transferrin (100 μg/ml), selenium (30 n*M*), putrescine (60 μ*M*), progesterone (20 n*M*), and penicillin–streptomycin (0.5 U/ml–0.5 μg/ml). Concentrated serum-free COS media containing the different trophic factors were diluted to appropriate concentrations and added to the cells at the time of plating. Cultures were grown for 4 or 7 days without changing media or supplementing the factors.

Catalytic Assays

Basal forebrain cultures were assayed for changes in ChAT activity by measuring incorporation of [^{14}C]-choline into [^{14}C]acetylcholine (10). Briefly, cells were harvested in 10 m*M* EDTA with 0.5% Triton and incubated with sodium phosphate (50 m*M*, pH 7.4), EDTA (20 m*M*), NaCl (300 m*M*), choline bromide (8 m*M*), physostigmine (0.1 m*M*), and [^{14}C]acetyl-CoA (0.2 m*M*) for 1 hr at 37°C. The assay was stopped with the addition of 10 m*M* sodium phosphate (pH 7.4) and 2 ml acetonitrile with 10 mg tetraphenylboron and counted in a liquid scintillation counter (Beckman) using a toluene scintillant.

Histochemical Procedures

(a) For acetylcholinesterase histochemistry, cultures were incubated in a 50 m*M* acetate buffer with 4 m*M* acetylthiocholine, 2 m*M* copper sulfate, 10 m*M* glycine, and 0.2 m*M* ethopropazine to inhibit nonspecific esterases. Cultures were then rinsed, exposed to 1.25% Na_2S, rinsed again, and exposed to 1% $AgNO_3$ (17). Labeled cells were counted in 2.5% of the area of the dish using a Zeiss Axiovert microscope.

(b) For tyrosine hydroxylase (TH) immunocytochemistry, cultures were fixed in 10% formalin and exposed to anti-TH antiserum. Cells were visualized using the avidin–biotin technique for immunoperoxidase staining. After incubation with biotinylated anti-rabbit IgG, cultures were exposed to the ABC reagent (Vector Laboratories). Cells were visualized by the 3,3-diaminobenzidine reaction product. All labeled cells in the dish were counted.

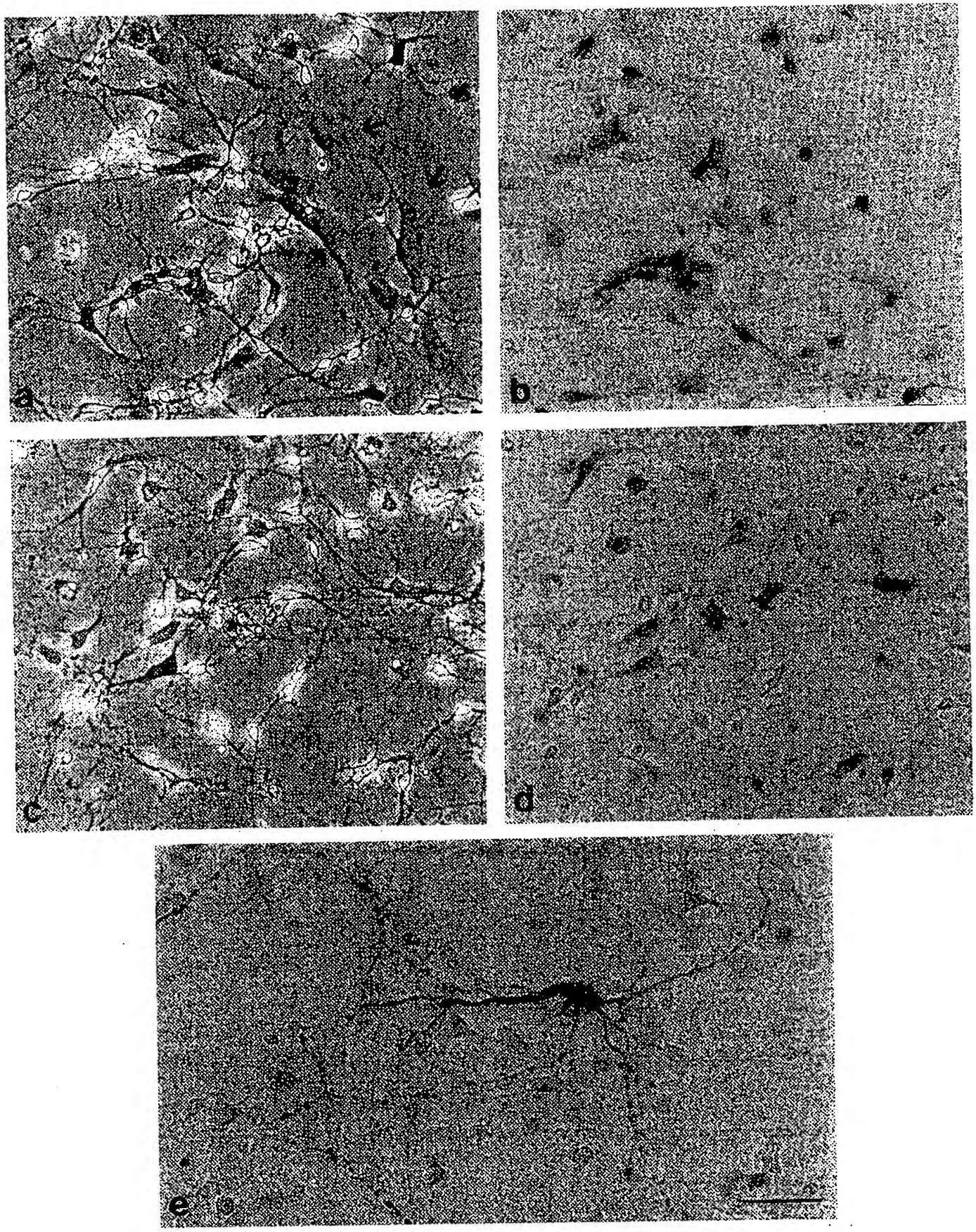
Production of Neurotrophins

The protein coding regions of the sequences for rat NGF, BDNF, NT-3, and *Xenopus* NT-4 were cloned into the expression vector pXM, a plasmid which is suitable for transient expression in COS cells (21). These cells were transfected with plasmids using the DEAE-dextran/chloroquine technique (29). Parallel transfections with an unrelated plasmid containing the β-galactosidase gene served as control. One day after transfection the medium was changed to a serum-free medium, and the cells were allowed to grow for 3 days. At this time the medium conditioned with the factors produced and secreted by the transfected COS cells was collected and concentrated 100× in Amicon concentrating tubes such that proteins larger than 10 kDa were retained. COS cells transfected with the control plasmid were assayed for β-galactosidase activity to assess the efficiency of transfection (20). The amount of trophic factor protein produced was assessed by growing parallel plates in the presence of [^{35}S]cysteine (22). The labeled conditioned COS media was then analyzed by SDS–polyacrylamide gel electrophoresis. The gels were treated with EnHance (NEN), dried, and subjected to autoradiography. Autoradiograms were analyzed using a Shimadzu densitometer. The amount of trophic factor produced was assessed by the amount of novel protein appearing in the transfected media migrating at approximately 13 kDa. Further, the amount of bioactive trophic factor produced by the COS cells was assessed by bioassay on peripheral ganglia and estimation of biological units in induction of neurite outgrowth (7). COS cell transfection routinely yielded in the range of 100 ng/ml of NGF and NT-4, and 20–50 ng/ml of BDNF and NT-3.

RESULTS

Neurotrophic Influences on the Locus Coeruleus

Trophic support of noradrenergic neuronal survival was monitored in the locus coeruleus (LC). Dissociated LC cultures from E16 rat fetuses were grown in serum with concentrated COS media containing 10–50 ng/ml of the different neurotrophins, or with concentrated media from cells transfected with a control plasmid. Cells were labeled immunocytochemically with antiserum directed against tyrosine hydroxylase (Fig. 1e). The number of TH-positive cells surviving after 1 week in culture increased twofold in the presence of NT-3 or


a
b
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e

NT-4 (Fig. 2), providing the first demonstration of neurotrophic influences in the locus coeruleus.

Cholinergic Cell Survival in Basal Forebrain Cultures

To determine whether cholinergic neuron survival was influenced by the different neurotrophins, E16 basal forebrain dissociated cell cultures were grown with NGF, BDNF, NT-3, or NT-4. Cholinergic neurons were labeled by acetylcholinesterase histochemistry (Figs. 1a–1d). The addition of 10–50 ng/ml of COS cell-produced BDNF, NT-3, or NT-4 elicited significant increases in cholinergic cell number (Fig. 3), suggesting a possible role in supporting neuronal survival. In contrast, NGF did not alter the number of cholinergic neurons in BF cultures. To ascertain whether COS cell medium may have masked an effect, 100 ng of purified 2.5S NGF was compared to COS NGF. Neither NGF preparation influenced cholinergic cell number (Fig. 3).

To assess whether COS cell-produced NGF had biological activity in the BF cultures, effects on ChAT activity were examined. COS NGF elicited a 2-3-fold increase in ChAT activity (Fig. 4), confirming previous results with purified NGF, and suggesting that this neurotrophin influences cholinergic function, but not neuronal survival in developing basal forebrain neurons in culture.

Influence of Neurotrophins in Pure Neuronal BF Cultures

BDNF, NT-3, and NT-4 influenced cholinergic neurons in BF cultures grown in the presence of serum as above. To ascertain whether the effects of these neurotrophins was exerted directly on the cholinergic neurons, cultures were grown under serum-free conditions, yielding a virtually pure neuronal population (Figs. 1c–1d). These cultures contained fewer than 5% glia. Using this preparation, BDNF, NT-3, and NT-4 elicited significant twofold increases in ChAT activity (Fig. 5), suggesting that the neurotrophins acted directly on the neuronal population.

DISCUSSION

In these studies, we have found that noradrenergic neurons of the locus coeruleus, which are insensitive to NGF (5, 34), respond to other neurotrophins, significantly increasing noradrenergic cell survival. Survival of LC neurons is known to be increased by hippocampal neurons in culture (37), suggesting that the target elaborates trophic factors. Specifically, both NT-3 (7, 9, 35), and NT-4 (unpublished data) are expressed in the hippocampus and appear to provide trophic support for LC neurons. The trophic actions of NT-3 in the locus coeruleus may be mediated by interactions with trkC, a receptor specific for NT-3 (27), which is expressed in the LC in adult rats (32). In contrast, NT-4 has been shown to activate trkB (23), suggesting that this receptor may be expressed in the LC as well.

Our studies also indicated that BDNF, NT-3, and NT-4 fostered BF cholinergic neuronal survival and function. We were able to dissociate effects on neurotransmitter parameters from those on cell survival. NGF, though eliciting increases in ChAT activity, failed to influence cholinergic cell survival, in contrast to the effects of BDNF, NT-3, and NT-4. The role of NGF in mediating cholinergic function but not survival indicates that the different neurotrophins may be highly specialized in their mechanisms of action. The complex trophic interactions require the presence of appropriate receptors on responsive neurons. The low affinity NGF receptor, p75, which binds all the neurotrophins, has been localized to the basal forebrain (44, 12), as have high affinity binding sites (4). The trk protooncogene product, which binds NGF (24, 18), and trkB, which binds BDNF (25, 40) and NT-4 (23), are both expressed in the adult rat basal forebrain (32). Moreover, trk mRNA has been detected in the E18 rat BF (8).

Both the locus coeruleus and basal forebrain innervate the hippocampus, a rich source of neurotrophic factors. Neurotrophin-3, which is synthesized in the hippocampus (9, 35), supported survival of both BF cholinergic neurons and LC noradrenergic neurons, indicating that a mutual target may synthesize a common trophic factor to support distinct neuronal pathways. Although recent data has demonstrated the presence of neurotrophins in the local environment of some responsive neurons, the absence of BDNF and NT-3 from the basal forebrain (11, 12) and locus coeruleus (8) suggests a possible target-derived mode of action with the hippocampus as the source of trophic support.

NT-4 also significantly increased both noradrenergic and cholinergic survival, as well as influencing ChAT activity. The trophic effects of NT-4 reported in this study were seen using recombinant NT-4 from *X. laevis*. In bioassays on peripheral sensory neurons from dorsal root and nodose ganglia, *Xenopus* and rat NT-4 have identical bioactivity (14, 23). Moreover, *Xenopus* and rat NT-4 are indistinguishable in receptor activation, eliciting specific tyrosine phosphorylation of trkB (23, and unpublished results). Thus, the *Xenopus* NT-4 is

FIG. 1. Dissociated cultures from the E16 embryonic rat brain. Basal forebrain neurons grown in either serum (a, b) or serum-free (c, d) conditions were labeled by acetylcholinesterase histochemistry (a–d). Cultures are shown using phase-contrast (a, c) or bright-field (b, d) microscopy. Arrows in (a) indicate flat support cells. Note absence of flat cells in c. A noradrenergic neuron from the locus coeruleus (e) was labeled immunocytochemically using anti-TH antiserum. Size bar in e is 50 μm and is the same for all panels.

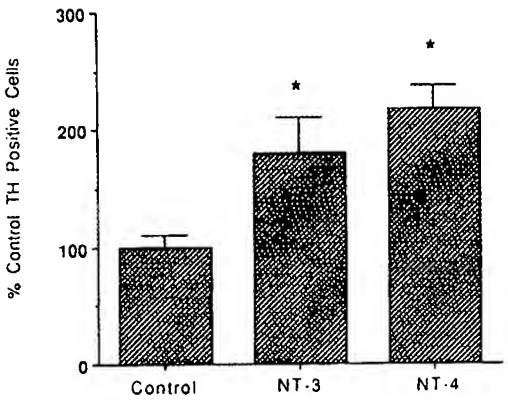

FIG. 2. Effects of NT-3 and NT-4 on TH neuron survival. Dissociated locus coeruleus cultures were grown in serum-containing media in the presence of COS-produced NT-3, NT-4, or control. TH-positive cells were counted and analyzed by analysis of variance. Data are shown as mean percentage control ± SEM from four independent experiments. Control LC cultures contained 121 ± 18 TH-positive neurons per dish. $^*P < 0.05$.

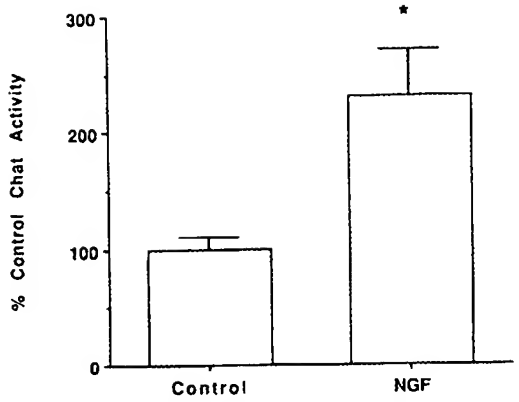

FIG. 4. Effect of COS-produced NGF on cholinergic function. ChAT activity was assayed in E16 BF dissociated cultures grown in serum and exposed to COS NGF or control in 10 independent experiments. $^*P < 0.05$.

capable of activating the rat trkB receptor, supporting the results obtained in the present study. Our observations on the trophic effects of NT-4 raise intriguing possibilities concerning the newly discovered NT-5, which appears to be the human homologue of *Xenopus*/viper NT-4 (3, 23). NT-5 has been detected in the adult rat brain (3), particularly in the cortex (unpublished results), another target common to both the basal forebrain and locus coeruleus.

The recombinant neurotrophins used in this study were derived from conditioned media from transfected COS cells without further biochemical purification. Similar amounts of the different neurotrophins were used in each assay, as assessed by quantification of autoradiograms from SDS–polyacrylamide gels of media from metabolically labeled COS cells. COS-produced NGF and purified 2.5S NGF elicited identical trophic responses in basal forebrain, indicating that the preparations have specific trophic activity. Moreover, no effects were seen with conditioned media from mock-transfected cells.

In sum, we have defined neurotrophic influences in two critical brain regions, the basal forebrain and locus coeruleus. Specific neurotrophins such as NT-3 and NT-4 enhanced survival of both neuronal populations. NGF, which has no effect on LC neurons, influenced the BF by increasing cholinergic function but not survival, in contrast to BDNF, NT-3, and NT-4. The discovery that neuronal survival in both the basal forebrain and locus coeruleus is affected by common target-derived trophic factors provides a possible mechanism by which

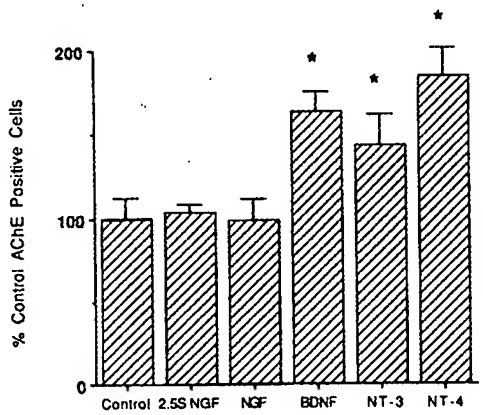

FIG. 3. Influence of neurotrophins on cholinergic neuron survival. Basal forebrain dissociated cell cultures were grown in serum-containing media in the presence of either control COS media, COS-produced neurotrophins, or purified 2.5S NGF. Acetylcholinesterase-positive cells were counted in three independent experiments and analyzed by ANOVA. Data are expressed as the mean percentage control ± SEM. $^*P < 0.05$.

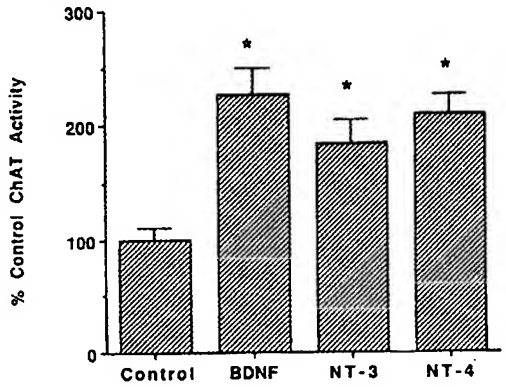

FIG. 5. Effects of neurotrophins in pure neuronal BF cultures. ChAT activity was assayed in basal forebrain dissociated cultures grown under serum-free conditions in the presence of COS control, BDNF, NT-3, or NT-4 concentrated media. Data are expressed as mean percentage control ± SEM from six independent experiments. $^*P < 0.05$.

these distinct populations undergo necrosis in a single degenerative disorder such as Alzheimer's disease.

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